# STUDIES ON COMPLEMENT FIXATION

## II. THE VELOCITY OF FIXATION OF COMPLEMENT IN THE WASSERMANN TEST *

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The marked difference of opinion among workers with regard to the time and temperature of fixation of complement in the Wassermann test can perhaps best be illustrated by the fact that two recently standardized Wassermann procedures recommend, in one case,[1] a fixation period of 40 minutes in the water bath and, in the other,[2] either 18 hours in the icebox or 3 to 4 hours in the icebox plus 1 hour in the water bath. The methods of fixation employed by different workers known to the writers embrace 30, 40 or 60 minutes in the water bath, 2 hours in the icebox plus 30 minutes in the water bath, and 4, 8, 10, 12 or 18 hours in the icebox. That all these methods give a relatively high degree of correct results is, in our opinion, not due to the fact that they are all correct, but rather to the nature of the Wassermann test. The majority of syphilitic serums possess such marked complement binding power that fixation periods ranging from 5 to 15 minutes are frequently quite ample. Simon[3] has indeed utilized this fact in his Wassermann tests, using for each test 2 fixation periods, 5 minutes and 1 hour, in the water bath. If a given test is positive after 5 minutes, he obviously needs go no further; if negative, the results are based on the hour fixation period. It is with the weakly positive serum that the method of fixation plays an important rôle. A longer fixation period would obviously increase the fixability of such serum. In attempting to prolong their fixation unduly, however, a practical difficulty presents itself. Complement gradually deteriorates at any temperature, and there is danger of confusing specific fixation with complement deterioration.

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* A preliminary report by Kahn, R. L.: Soc. for Exper. Biol. and Med., 1921, 18, p. 168.

[1] Hinton, W. A.: Jour. Syph., 1920, 4, p. 598.

[2] Kolmer, J. A.: Matsunami, T., and Trist, M. E.: Jour. Syph., 1921, 5, p. 63. This paper as well as one by Dean, Jour. Path. & Bacteriol., 1917, 21, p. 193, give an extensive review of the literature.

[3] Jour. Amer. Med. Assn., 1917, 72, p. 1535.

Perhaps the outstanding feature of the generally accepted views of fixation is that short periods (one-half to one hour) at water bath temperature give parallel results with prolonged periods (4 to 18 hours) at icebox temperature. The logic of this assumption is clear. The complement-fixation reaction being biologic in nature, it presumably takes place more rapidly at 37.5 C. than at lower temperatures. That this assumption does not hold true in complement fixation with purified proteins has been shown by one of us in a previous paper.[4] The rate of fixation of complement with immune rabbit serum and protein antigens was found to be similar at water bath, room or icebox temperature, with a slight tendency for stronger fixation at the latter temperature. It was further shown that the maximum degree of fixation was obtained after 4 hours at icebox temperature. To determine whether these results are applicable to complement-fixation tests with syphilitic serum and the usual Wassermann antigens has been the aim of this paper.

## EXPERIMENTS

The complement-fixation tests were carried out with a sheep cell system, employing 2 units of complement, 2 units of amboceptor and 4 to 5 units of antigen. These ingredients, as well as the sheep cell suspension, were used in 0.1 c.c. quantities. The syphilitic serums used in these experiments were positive Wassermann serums left over from those sent to this laboratory for examination. These serums were used in every case in the following gradations: 0.01, 0.007, 0.004, 0.003, 0.002, 0.001, 0.0005, 0.0003, and 0.0001 c.c.

Six antigens were employed: (1) an alcoholic extract of beef hearts previously freed from ether soluble lipoids; (2) the same antigen, cholesterinized; (3) a crude alcoholic extract of guinea-pig hearts; (4) a Noguchi antigen; (5) a cholesterinized antigen of human hearts; and (6) a crude alcoholic extract of beef hearts.

## THE PREPARATION OF THE ANTIGENS

1. The alcoholic extract of beef hearts was prepared according to the method described by Neymann and Gager.[5] Briefly, fresh beef hearts were freed from fat, fiber and blood vessels and then ground and dried. The dried material was then extracted 4 or 5 times with ether. These extractions were carried out for several days at a time in the icebox

[4] Kahn, R. L.: Jour. Exper. Med., 1921, 34, p. 217.

[5] Jour. Immun, 1917, 2, p. 573. Compare Ecker, E. E. and Sasani, K.: Jour. Infect. Dis., 1919, 24, p. 174.

and continued until the supernatant ether showed no coloring matter, the ether being discarded in every case. The beef heart was then completely freed from ether by drying, placed in a flask, and absolute alcohol added in such proportions that a layer of fluid about 1 inch high covered the dried material. The alcoholic extract was ready for use after about 2 weeks' extraction.

2. The cholesterinized antigen was the one just described, except that it was half saturated with cholesterol, or, what is equal to the same thing, 0.4 gm. of cholesterol was added to every 100 c c.

3. The guinea-pig antigen was prepared by extracting guinea-pig hearts, previously cut into small pieces and dried between filter paper, in absolute alcohol. The extraction was carried out for several months in the icebox. This antigen was kindly given us by Mr. John Koopman, serologist of the New York City Health Department.

4. The Noguchi antigen was prepared from beef hearts in the manner described by Noguchi.[6]

5. The cholesterinized antigen of human hearts was prepared similarly to the guinea-pig heart antigen except that it was half saturated with cholesterol.

6. The crude alcoholic extract of beef hearts was also prepared similarly to the guinea-pig heart antigen.

These antigens were titrated for their antigenic, anticomplementary and hemolytic properties in the usual manner. Five times the quantity of these antigens employed in the tests were neither anticomplementary nor hemolytic.

## THE EFFECT OF TEMPERATURE ON THE VELOCITY OF FIXATION OF COMPLEMENT IN THE WASSERMANN TEST

The fixation periods employed were 0, 5, 15, and 30 minutes and 1, 2, 3, 4, 5, 6 and frequently 7 hours. The temperatures were icebox (8 to 12 C.), room (18 to 23 C.) and water bath (37.5 C.). The tests were carried out in the usual manner, employing the various gradations of serum, 0.1 c c of antigen, 0.1 c c (2 units) of complement and 0.1 c c of salt solution. After a given fixation period, 0.1 c c of the standard sheep cell suspension (5%) and 0.1 c c hemolysin (2 units) were added and incubated in the water bath at 37.5 C. for about 15 minutes, when the serum and antigen controls would be completely hemolyzed. All readings were made after keeping the tubes in the icebox over night.

[6] Serum Diagnosis of Syphilis, Ed. 2, p. 79.

The fixation experiments at water-bath and room temperatures were not extended beyond 2 hours, in view of the marked deterioration of complement after prolonged exposure at these temperatures.

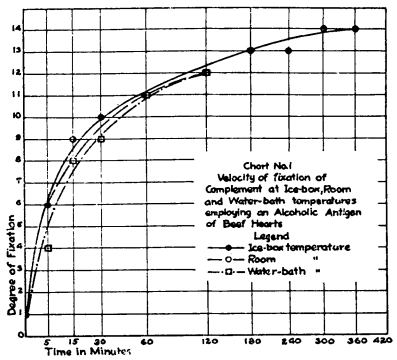
TABLE 1

VELOCITY OF FIXATION OF COMPLEMENT AT ICEBOX, WATER-BATH, AND ROOM TEMPERATURES
Tests with Alcoholic Antigen of Beef Heart

| Fixation | | Wassermann Positive Syphilitic Serum (c c) | | | | | | | | | Number of Positive Signs Denoting Degree of Fixation |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Period | Temperature | 0.01 | 0.007 | 0.004 | 0.003 | 0.002 | 0.001 | 0.0005 | 0.0003 | 0.0001 | |
| 0 Min. | ..... | 1* | — | — | — | — | — | — | — | — | 1 |
| 5 Min. | Icebox..... | 3 | 2 | 1 | — | — | — | — | — | — | 6 |
| | Water bath..... | 2 | 1 | 1 | — | — | — | — | — | — | 4 |
| | Room..... | 3 | 2 | 1 | — | — | — | — | — | — | 6 |
| 15 Min. | Icebox..... | 4 | 3 | 1 | 1 | — | — | — | — | — | 9 |
| | Water bath..... | 3 | 3 | 1 | 1 | — | — | — | — | — | 8 |
| | Room..... | 4 | 3 | 1 | 1 | — | — | — | — | — | 9 |
| 30 Min. | Icebox..... | 4 | 4 | 1 | 1 | — | — | — | — | — | 10 |
| | Water bath..... | 4 | 3 | 1 | 1 | — | — | — | — | — | 9 |
| | Room..... | 4 | 3 | 1 | 1 | — | — | — | — | — | 9 |
| 60 Min. | Icebox..... | 4 | 4 | 2 | 1 | — | — | — | — | — | 11 |
| | Water bath..... | 4 | 4 | 2 | 1 | — | — | — | — | — | 11 |
| | Room..... | 4 | 4 | 2 | 1 | 1 | — | — | — | — | 12 |
| 2 Hrs. | Icebox..... | 4 | 4 | 2 | 1 | 1 | — | — | — | — | 12 |
| | Water bath..... | 4 | 4 | 2 | 1 | 1 | — | — | — | — | 12 |
| | Room..... | 4 | 4 | 2 | 1 | 1 | — | — | — | — | 12 |
| 3 Hrs. | Icebox..... | 4 | 4 | 2 | 2 | 1 | — | — | — | — | 13 |
| 4 Hrs. | Icebox..... | 4 | 4 | 2 | 2 | 1 | — | — | — | — | 13 |
| 5 Hrs. | Icebox..... | 4 | 4 | 3 | 2 | 1 | — | — | — | — | 14 |
| 6 Hrs. | Icebox..... | 4 | 4 | 3 | 2 | 1 | — | — | — | — | 14 |

* 4 = ++++, 3 = +++, 2 = ++, 1 = +, and — = negative.

Forty-five different syphilitic serums were used in these experiments, and because of the general uniformity of the findings only 2 tables will be given. Table 1 gives the results of one experiment carried out with the alcoholic extract antigen of beef hearts.



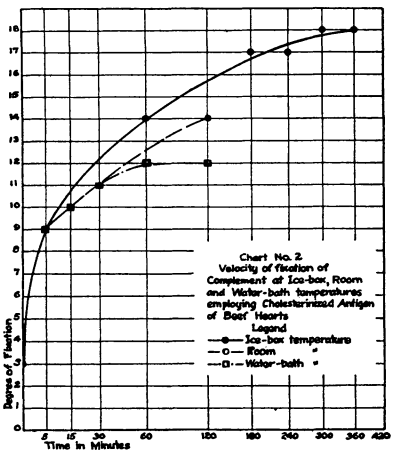

It is evident from table 1 that the velocity of fixation of complement is practically the same at icebox, room, and water bath temperatures. The degree of fixation is reduced to a numerical value by adding the

number of plus signs after each fixation period. Chart 1 is based on the findings recorded in this table. It will be noted that the hour divisions on the time axis (abscissae) are so spaced that each one is equal to the two thirds of the power of the preceding division. In this

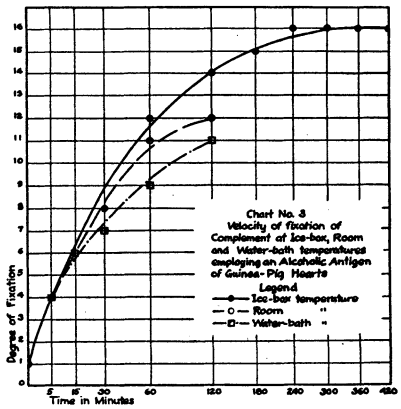
TABLE 2

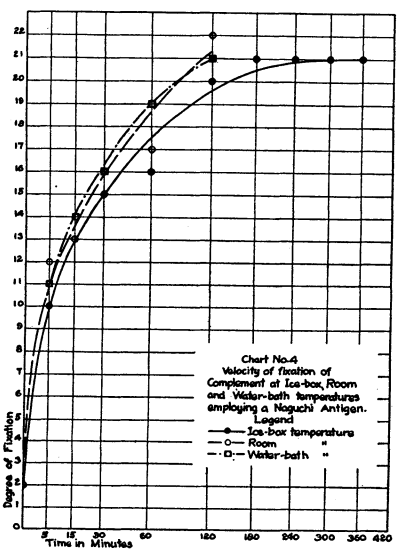
VELOCITY OF FIXATION OF COMPLEMENT AT ICEBOX, WATER-BATH, AND ROOM TEMPERATURES
Tests with Cholesterinized Antigen of Beef Heart

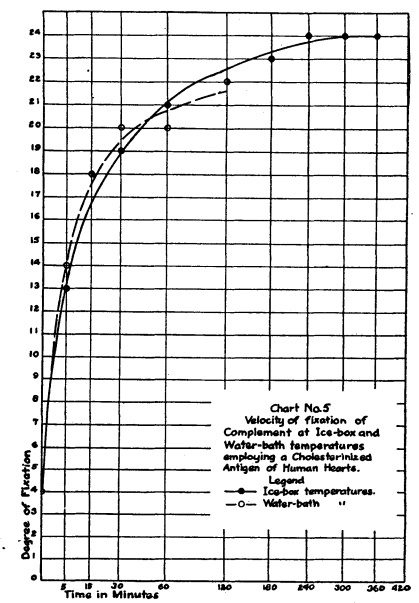
| Fixation | | Wassermann Positive Syphilitic Serum (c c) | | | | | | | | | Number of Positive Signs Denoting Degree of Fixation |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Period | Temperature | 0.01 | 0.007 | 0.004 | 0.003 | 0.002 | 0.001 | 0.0005 | 0.0003 | 0.0001 | |
| 0 Min. | ..... | 2 | 1 | — | — | — | — | — | — | — | 3 |
| 5 Min. | Icebox..... | 4 | 3 | 1 | 1 | — | — | — | — | — | 9 |
| | Water bath..... | 4 | 3 | 1 | 1 | — | — | — | — | — | 9 |
| | Room..... | 4 | 3 | 1 | 1 | — | — | — | — | — | 9 |
| 15 Min. | Icebox..... | 4 | 4 | 1 | 1 | — | — | — | — | — | 10 |
| | Water bath..... | 4 | 4 | 1 | 1 | — | — | — | — | — | 10 |
| | Room..... | 4 | 4 | 1 | 1 | — | — | — | — | — | 10 |
| 30 Min. | Icebox..... | 4 | 4 | 2 | 1 | — | — | — | — | — | 11 |
| | Water bath..... | 4 | 4 | 2 | 1 | — | — | — | — | — | 11 |
| | Room..... | 4 | 4 | 2 | 1 | — | — | — | — | — | 11 |
| 60 Min. | Icebox..... | 4 | 4 | 3 | 2 | 1 | — | — | — | — | 14 |
| | Water bath..... | 4 | 4 | 2 | 1 | — | — | — | — | — | 12 |
| | Room..... | 4 | 4 | 3 | 1 | — | — | — | — | — | 12 |
| 2 Hrs. | Icebox..... | 4 | 4 | 3 | 2 | 1 | — | — | — | — | 14 |
| | Water bath..... | 4 | 4 | 2 | 1 | 1 | — | — | — | — | 12 |
| | Room..... | 4 | 4 | 3 | 2 | 1 | — | — | — | — | 14 |
| 3 Hrs. | Icebox..... | 4 | 4 | 4 | 2 | 2 | 1 | — | — | — | 17 |
| 4 Hrs. | Icebox..... | 4 | 4 | 4 | 2 | 2 | 1 | — | — | — | 17 |
| 5 Hrs. | Icebox..... | 4 | 4 | 4 | 3 | 2 | 1 | — | — | — | 18 |
| 6 Hrs. | Icebox..... | 4 | 4 | 4 | 3 | 2 | 1 | — | — | — | 18 |




way it was possible to plot 5, 15 and 30 minute values on a relatively large scale and at the same time keep the width of the chart within small limits.


Degree of Fixation
Time in Minutes
5 15 30 60 120 180 240 300 360 420
Chart No. 3
Velocity of fixation of Complement at Ice-box, Room and Water-bath temperatures employing an Alcoholic Antigen of Guinea-Pig Hearts
Legend
Ice-box temperature
Room "
Water-bath "


Degree of Fixation
Time in Minutes
5 15 30 60 120 180 240 300 360 420
Chart No.4
Velocity of fixation of Complement at Ice-box, Room and Water-bath temperatures employing a Noguchi Antigen.
Legend
Ice-box temperature
Room "
Water-bath "


Degree of Fixation
Time in Minutes
5 15 30 60 120 180 240 300 360 420
Chart No.5
Velocity of fixation of Complement at Ice-box and Water-bath temperatures employing a Cholesterinized Antigen of Human Hearts.
Legend
Ice-box temperatures.
Water-bath "

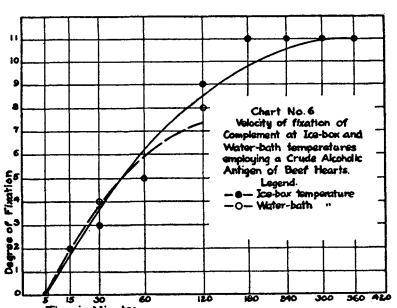
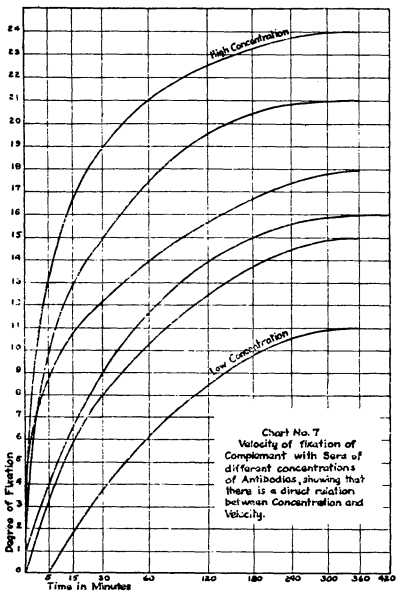

Degree of Fixation
Time in Minutes
5 15 30 60 120 180 240 300 360 420
Chart No. 6
Velocity of fixation of Complement at Ice-box and Water-bath temperatures employing a Crude Alcoholic Antigen of Beef Hearts.
Legend.
Ice-box temperature
Water-bath "

Table 2 and chart 2 give the results of a similar experiment with the cholesterinized antigen of beef heart. The tendency for somewhat stronger fixation at icebox temperature is illustrated by this experiment. Charts 3 to 6, inclusive, represent experiments with different antigens. With the exception of the Noguchi antigen chart, all show the same tendency, namely, either equal fixation at all temperatures or a tendency for stronger fixation at icebox temperature. In the case of the Noguchi antigen (chart 4), however, the tendency for somewhat stronger fixation at room and water bath temperatures than at icebox




temperature is indicated. In several instances icebox temperature gave somewhat stronger fixation with this antigen also In most cases, the tendency was as indicated in this chart, except that fixation at room temperature was, as a rule, weaker than at water bath temperature.

Another element brought out by these charts is that 4 hours' fixation at icebox temperature approaches the maximum amount of complement that serum and antigen, in the quantities employed in these experiments, are capable of binding. That this does not hold true in every case when employing a Noguchi antigen is indicated in chart 4. Even with this antigen, however, the amount of complement fixed after 4 hours' incubation in the icebox was, in most cases, somewhat greater than after 1 hour in the water bath.

It will be recalled that our complement-fixation studies with protein antigens [4] indicated that the rate of fixation of complement was determined by the concentration of antibodies in the immune serum. That the same holds true with syphilitic serums and Wassermann antigens is indicated in chart 7, which consists of 6 icebox fixation curves representing serums of different concentrations. A study of this chart reveals that a serum of high antibody concentration shows considerable fixation of complement immediately after mixing the ingredients and over 50% of fixation after an incubation period of 5 minutes. With serums of lesser antibody concentration, the curves rise less and less abruptly, and the first signs of fixation do not take place until after from 5 to 15 minutes' incubation.

This chart, as well as the preceding ones, speak against ½ to 1 hour fixation periods at water bath temperature as well as prolonged fixation periods, such as 12 to 18 hours, at icebox temperature. The employment of a 4-hour fixation period at icebox temperature in the Wassermann test would appear, from our results, to be a dependable procedure.

The well-known tendency of cholesterinized antigens to pick up occasional false positive reactions, however, puts them in a class by themselves. And with the routine Wassermann tests carried out in this laboratory with this antigen, a 4-hour fixation period is, in our opinion, unsafe. We employ this fixation period with our alcoholic antigen tests, but, in the case of the cholesterinized antigen tests, a 1-hour fixation period at icebox temperature is resorted to. In view of the relatively sharper binding power of cholesterinized antigens compared with alcoholic antigens when employed with the same serum, a 4-hour period with the latter antigens gives practically identical results with a 1-hour fixation period with cholesterinized antigens. The main advantage of icebox over water-bath fixation lies in the fact that, at the former temperature complement is practically preserved, while at the latter it deteriorates rapidly.

SUMMARY

The velocity of fixation of complement employing syphilitic serums and 6 different Wassermann antigens was studied. The periods of fixation were 0, 5, 15 and 30 minutes and 1, 2, 3, 4, 5, 6 and frequently 7 hours. The temperatures of fixation were water bath, room and icebox, and, in some cases, water bath and icebox.

It was observed that the velocity of fixation of complement is not markedly affected by temperatures ranging between water bath and

icebox. The tendency for slightly stronger fixation at icebox temperature compared with that of the water bath was noted with all antigens, except the Noguchi. The latter antigen showed a tendency for somewhat stronger fixation at water bath temperature.

It was also observed that a fixation period of 4 hours at icebox temperature approaches the maximum amount of fixation of complement with all antigens, including the Noguchi, although the latter in a few cases showed slightly more fixation after 1 hour in the water bath than after 4 hours in the icebox.

Finally, it was shown that the velocity of fixation of complement is directly proportional to the concentration of antibodies in the syphilitic serums.